PPD®	Document Number: **GCL-LAB-0936**
	Revision Number: **04**
	Effective Date: **01JUN2019**
Global Central Labs	Page 1 of 24

Title: Standard Process for PBMC, BMMC, PMNC and Leucocyte (WBC) Isolations

Location:	☒ Belgium	☒ China - Shanghai	☒ Singapore	☒ United States
Lab Sub-Unit:				
☐ General (includes AP)	☐ Anatomic Pathology	☐ Centaur/Immulite	☐ Cobas Chemistry	☐ Cobas Immunoassay
☐ General (excludes AP)				
☐ Chromatography/HPLC	☐ Coagulation	☐ Flow Cytometry	☐ Hematology	☐ HGA1C/HPLC/TOSOH
☐ EIA	☐ Microbiology	☐ Molecular	☒ PBMC	☐ Phadia
☐ POC	☐ R&D	☐ Special Chemistry	☐ Urinalysis	

Supersedes: GCL-LAB-0936r03	**Author: Bernelyn J. Tranos**
	Title: Section supervisor

CHANGE HISTORY		
Revision No.	**Change Details**	**Training Required?**
00	Initial version	☒YES ☐NO
01	Updated version includes supervisor review of the processing worksheets, with the defined review criteria and frequency. Competency assessment for the techs including the post thaw competency	☒YES ☐NO
02	Added Aliquot Review, update list of reagents and specimen section on how to use the ISR module.	☒YES ☐NO
03	Format update. Leucocyte isolation procedure and Dried blood spot procedure added in section 12. Reagents list and Attachment A updated. Elaborate on the aliquot review process. Adding PMNC and Leucocyte isolations process. Updating attachment.	☒YES ☐NO
03	Administrative Update 25APR2019: Title had changed from r02 to r03 but was not updated on r03's change history. FROM *Standard Process for PBMC and BMMC Isolations* TO *Standard Process for PBMC, BMMC, PMNC and Leucocyte (WBC) Isolations*	☐YES ☒NO
04	Update QC sterility check, add incubation temperature and modify attachment Reagent QC log to add start/end date. Update references, remove Client name. Add China-SH location.	☒YES ☐NO

Title: Standard Process for PBMC, BMMC, PMNC and Leucocyte (WBC) Isolations

1. Purpose

To provide a standardized procedure using regulatory requirements for performing assay testing.
To provide a standardized procedure, using Clinical and Laboratory Standards Institute guidelines for isolation and freezing of PBMCs, BMMCs (or BBMC), PMNCs (Granuloctyes) and WBCs. **This SOP is intended to be PPDs standard procedure. Each technologist must be trained in accordance to the general procedure.**

2. Scope

All PPD Global Central Laboratories.

3. Assay Information

External Name	MTM Number	Lab Assay Name	FDA Approval
PBMC Isolation	18259	Peripheral Blood Mononuclear Cells Isolation_ (PBMC)	N/A
PBMC Isolation	4476	Peripheral Blood Mononuclear Cells Isolation (PBMC)	N/A
PBMC, viability	1612	Peripheral Blood Mononuclear Cells, Viability (PBMC)	N/A
Peripheral Blood Mononuclear Cell Isolation (PBMC)_Standard Process	11139	Peripheral Blood Mononuclear Cell Isolation (PBMC)_Standard Process	N/A
PMNC Isolation	21979	Polymorphonuclear Cells	N/A
PMNC, viability	22000	Polymorphonuclear Cells, Viability	N/A

4. Key Definitions

Term	Definition

PBMC	Peripheral blood mononuclear cells – devoid of most neutrophils, basophils and eosinophils.
BBMC	Blood Bone marrow mononuclear cells- cell population includes hematopoietic progenitor cells, lymphoid cells (lymphocytes, plasma cells), monocytes, and macrophages.
BMMC	Bone marrow mononuclear cells-(Same as BBMC)
PMNC	Polymorphonuclear Cells, Granulocytes, Composed of Neutrophils, Eosinophils, Basophils
BME	2-Mercaptoethanol
Buffy coat	The white cell layer directly above the Red Cells of a whole blood specimen that has been spun. Consists of all white blood cells.
CPT	Cell Preparation Tubes
DMSO	Dimethyl sulfoxide
PBS	Phosphate buffered saline
FBS	Fetal Bovine Serum
RCF	Relative centrifugal force; the centrifugal force acting on a rotating object expressed in terms of multiples of the gravitational force.
RPM	Revolutions per minute; a measurement used to express rotational speed centrifuges. It is the number of full rotations completed in one (1) minute.
RPMI	Roswell Park Memorial Institute
Leucocyte (WBC)	cells of the immune system involved in protecting the body against both infectious disease and foreign invaders

5. Special Safety Precautions

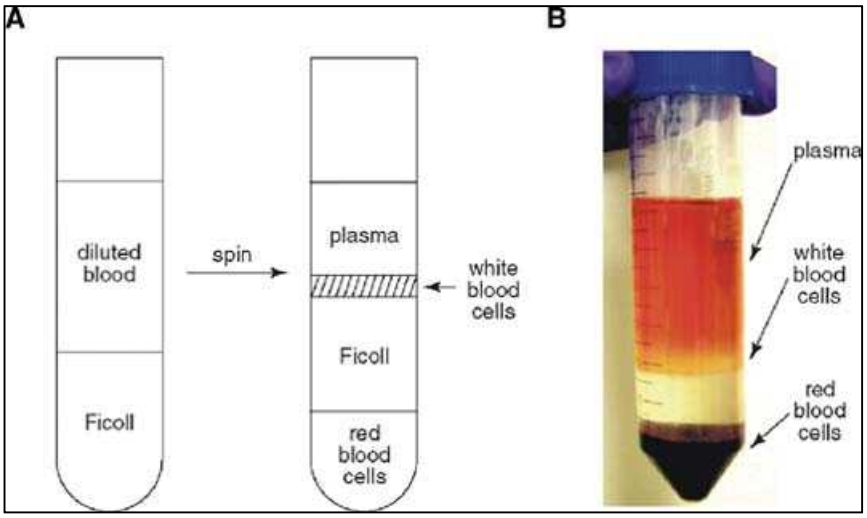
Standard safety precautions are required for handling laboratory reagents and samples. Entry to the laboratory requires appropriate PPE, at a minimum a fully closed lab coat, safety glasses, and gloves. Additional PPE may be required according to department and task performed. When handling samples, apply safety precautions as described in the Bloodborne Pathogen Exposure Plan and Chemical Hygiene Plan. When handling reagents and chemicals, always read the product label. For more information, consult the Safety Data Sheet. SDS information can be found online at www.3eonline.com. Contact your EHS representative for additional information as needed.
Cryogloves and face shield are required for handling Liquid Nitrogen.
All procedures should be done in a sterile environment.

6. Analytical Principle

- The isolation of peripheral blood mononuclear cells (PBMCs) from whole blood and Bone Marrow mononuclear cells (BMMCs) from bone marrow samples is an important pre-analytical step for many routine laboratory procedures including but not limited to flow cytometry, intracellular cytoplasmic cytokine staining (ICS), cytokine proliferation studies, genetic testing, and EliSpot analysis.

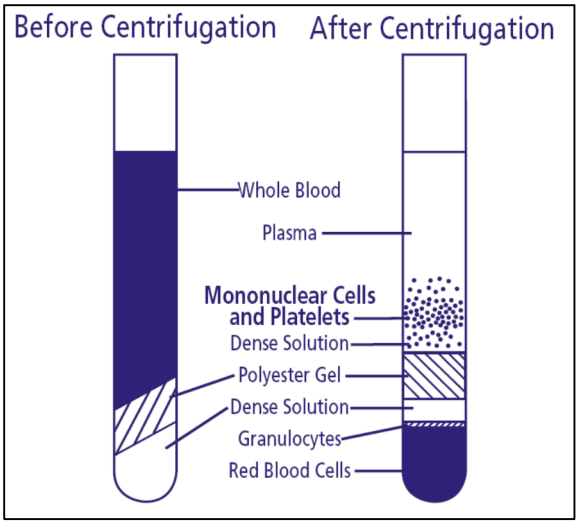
- Non-CPT- When human whole blood and/or bone marrow aspirates are layered over a density gradient fluid such as Ficoll-Paque Plus or Ficoll-Hypaque with a density of 1.077 and spun at 400 to 1200 x g (optimal 900 x g) for 20 to 30 minutes at room temperature the blood will separate into its cellular components with the mononuclear cells (lymphocytes and monocytes) readily available for harvesting. See figure 1.

Figure 1 Density Gradient Separation of Mononuclear Cells



- CPT- Isolation of mononuclear cells is the first step for many in vitro assays. In the Ficoll Hypaque method a liquid density gradient is created with Ficoll 400 and sodium metrizoate or sodium diatrizoate solution. Upon centrifugation the mononuclear cells will separate into a layer above this medium. See Figure 2.

Figure 2



7. Specimen Requirements

- **Plasma – If Applicable**

Preferred Sample Type(s)		Plasma collected from a specimen type specified by the client.
Alternative Sample Type(s)		N/A
Sample Collection		N/A
Sample Container		N/A
Transport Container		N/A
Sample Stability	**Ambient**	Room Temperature up to 72 hours. ▪ Optimal specimen **should** be received by the laboratory within 24 hours **and** processing **should** begin no later than 36 hours of specimen collection. ▪ Specimens received after 36 hours of collection but no later than 72 hours of collection (unless otherwise allowed in the sponsor specific worksheet) should be processed **but** a notation will be reported on the sponsor specific worksheet and an external comment is to be placed on the LIS.
	2-8°C	N/A
	-20°C	N/A
	-70°C	N/A
Volume Requirements		N/A
Special Handling		N/A
Rejection Criteria		Insufficient quantity, broken tubes, broken gel barrier, hemolyzed, clotted, specimen older than 72 hours. Isolated PBMC's that are to be stored in liquid nitrogen are to be placed into liquid nitrogen no more

	later than 48 hours after the cells are re-suspended in freezing media. If 72 hours is exceeded, approval from the client and PM is needed to store those PBMC's in liquid nitrogen. PBMC's may be stored into the laboratory liquid nitrogen tank, but only with supervisor approval.
Interfering Substances	N/A

- **PBMC and BMMC**

Preferred Sample Type(s)		Sodium Heparin, K2EDTA, K3EDTA, Na2EDTA, ACD, BCT, Lithium Heparin, CPT Sodium Heparin, CPT Sodium Citrate, and Streck Cell-Free DNA BCT for PBMCs and BBMCs.
Alternative Sample Type(s)		N/A
Sample Collection		N/A
Sample Container		N/A
Transport Container		N/A
Sample Stability	**Ambient**	Room Temperature up to 72 hours. ▪ Optimal specimen **should** be received by the laboratory within 24 hours **and** processing **should** begin no later than 36 hours of specimen collection. ▪ Specimens received after 36 hours of collection but no later than 72 hours of collection (unless otherwise allowed in the sponsor specific worksheet) should be processed **but** a notation will be reported on the sponsor specific worksheet and an external comment is to be placed on the LIS.
	2-8°C	N/A
	-20°C	N/A
	-70°C	N/A
Volume Requirements		N/A
Special Handling		N/A
Rejection Criteria		Insufficient quantity, broken tubes, broken gel barrier, hemolyzed, clotted, specimen older than 72 hours. Isolated PBMC's that are to be stored in liquid nitrogen are to be placed into liquid nitrogen no more later than 48 hours after the cells are re-suspended in freezing media. If 72 hours is exceeded, approval from the client and PM is needed to store those PBMC's in liquid nitrogen. PBMC's may be stored into the laboratory liquid nitrogen tank, but only with supervisor approval.
Interfering Substances		N/A

- PBMC and BBMC samples received in lab should be checked per screen ID. For samples without screen ID:

- Scan samples in ConneXion, ISR portal.
- View Patient Demographics and scan the sample and choose the Visit.
- It will then show the set of samples received for the patient – (extra step) the team also checks the CLS of the study to be sure of the # of tubes we're supposed to receive for each PBMC patient.

- **Leucocyte (WBC)**

Preferred Sample Type(s)		Sodium Heparin, K2EDTA, K3EDTA, Na2EDTA, ACD, BCT, Lithium Heparin, CPT Sodium Heparin, CPT Sodium Citrate, and Streck Cell-Free DNA BCT for PBMCs and BBMCs.
Alternative Sample Type(s)		N/A
Sample Collection		N/A
Sample Container		N/A
Transport Container		N/A
Sample Stability	**Ambient**	Room Temperature up to 72 hours. ▪ Optimal specimen **should** be received by the laboratory within 24 hours **and** processing **should** begin no later than 36 hours of specimen collection. ▪ Specimens received after 36 hours of collection but no later than 72 hours of collection (unless otherwise allowed in the sponsor specific worksheet) should be processed **but** a notation will be reported on the sponsor specific worksheet and an external comment is to be placed on the LIS.
	2-8°C	N/A
	-20°C	N/A
	-70°C	N/A
Volume Requirements		N/A
Special Handling		N/A
Rejection Criteria		Insufficient quantity, broken tubes, broken gel barrier, hemolyzed, clotted, specimen older than 72 hours. Isolated PBMC's that are to be stored in liquid nitrogen are to be placed into liquid nitrogen no more later than 48 hours after the cells are re-suspended in freezing media. If 72 hours is exceeded, approval from the client and PM is needed to store those PBMC's in liquid nitrogen. PBMC's may be stored into the laboratory liquid nitrogen tank, but only with supervisor approval.
Interfering Substances		N/A

8. Reagent and Media

Do not use reagents past manufacturer expiration. Do not mix reagents from different kits.

8.1. Reagent Information

Reagent	US Catalog Number	EU Catalog Number	SG Catalog Number	SHA Catalog Number	Vendor
Ficoll-Paque Plus density 1.077	45-001-737*	17-1440-02*	17-1440-02*	Not applicable	Fisher
Histopaque - 1077		10771* (6*100ml)	Not applicable	Not applicable	VWR
PBS (no Mg, no Ca, no Phenol Red)	14190-250* (500mL)	14190-169* (500mL)	14190-250* (500mL)	Not applicable	Invitrogen
RPMI1640 medium (no L-glutamine)	21870-092* (500mL)	11534-446* (500mL)	21870-092* (500mL)	Not applicable	Invitrogen
RPMI1640 medium (with L-glutamine)	21875-034* (500mL)	11530586* (500mL)	Not applicable	Not applicable	Invitrogen
RLT Buffer	79216 (220mL)	79216 (220mL)	79216 (220mL)	Not applicable	Qiagen
RNAprotect	76526 (250mL)	76526 (250mL)	76526 (250mL)	Not applicable	Qiagen
RNAlater	AM7020 (250mL)	AM7022 (50*1.5mL aliquots)	Not applicable	Not applicable	Thermofisher
BME (2-Mercaptoethanol or ß-Mercaptoethanol)	M6250 (100ML)	M6250 (100ML)	M6250 (100ML)	Not applicable	Sigma
CTL-Cryo ™ ABC Freezing kit	C.T.L. (CLTL-ABC) - CTL-Cryo™ ABC Freezing Kit (3 vials per kit, 10ml total volume)	Not applicable	C.T.L. (CLTL-ABC) - CTL-Cryo™ ABC Freezing Kit (3 vials per kit, 10ml total volume)	Not applicable	Immunospot
100x Penicillin/Streptomycin (5000 units Pen/5000	15070-063* (100 mL)	15070-063* (100 mL)	15070-063* (100 mL)	Not applicable	Invitrogen

µg Streptomycin) - Frozen					
100x L-glutamine (2902 mg/mL in 0.85% NaCl) - Frozen	25030-081* (100 mL)	11500626* (100 mL)	25030-081* (100 mL)	Not applicable	Invitrogen
1 M HEPES Buffer (238.3 g/L in distilled water)	15630-080	15630-056	15630-080	Not applicable	Invitrogen
Fetal Bovine Serum	10082-147*	10082-147*	10082-139* (100 mL)	Not applicable	Invitrogen
Dimethyl Sulfoxide (DMSO) (Tissue Culture Grade – sterile filtered)	D2650*	D2650*	D2650*	Not applicable	Sigma
RBC Lysis Buffer 10X	420301	10003412	420301	Not applicable	Biolegend
Trypan Blue 0.4% in 0.85% saline	15250-061	15250-061	15250-061	Not applicable	Invitrogen
Thioglycollate Broth (Medium)	– 221195 (Case of 10) – 221196 (Case of 100)	– 221195 (Case of 10) – 221196 (Case of 100)	Not applicable	Not applicable	BD
Accuspin System-Histopaque 1.077 (12mL tubes)	A6929	Not applicable	Not applicable	Not applicable	Sigma
Accuspin System-Histopaque 1.077 (50mL tubes)	A7054	Not applicable	Not applicable	Not applicable	Sigma
Isopropanol			563935-1L*	Not applicable	Sigma
Hanks Balanced Solution (HBSS)	H6648*	H6648*	14175-103*	Not applicable	Sigma/ Invitrogen

BSA (Bovine Serum Albumin)	A8412 (100mL)	A8412 (100mL)	A8412 (100mL)	Not applicable	Sigma
DMEM (Dulbecco's Modified Eagle Medium)	11885-084	11885-084	11885-084	Not applicable	Thermofisher / Invitrogen
Recovery Cell Culture Freezing Medium	12648-010	12648-010	Not applicable	Not applicable	Thermofisher
Synth-a-Freeze	10364330	10364330	A12542-01	Not applicable	Thermofisher / Invitrogen
Whatman® Protein Saver Card	WHA10534612 *	WHA10534612 *	Not applicable	Not applicable	Aldrich
Dextran	AAJ6378922*	AAJ6378922*	Not applicable	Not applicable	Fisher
Sodium Chloride	S7653*	S7653*	Not applicable	Not applicable	Sigma
IMDM, HEPES 500mL	11504556	11504556	Not applicable	Not applicable	Fisher
Human Serum albumin 20%solution	31020* (500mL)	31020* (500mL)	Not applicable	Not applicable	VWR
AB biowest human serum albumin	S4190* (100mL)	S4190* (100mL)	Not applicable	Not applicable	VWR
Benzonase Nuclease	70664*	70664*	70664*	Not applicable	VWR
Cryostor10	C2874	C2874	C2874	Not applicable	Sigma

*or equivalent

8.2. Reagent Preparation

Freezing medium: 90%FBS/10%DMSO	
Preparation	90% FBS + 10% DMSO
Storage	Label as s-FBS/DMSO with an expiration date of 30 days. Store at 4°C.
Freezing medium: 80%FBS/20%DMSO	
Preparation	80% FBS + 20% DMSO
Storage	Label as s-FBS/DMSO with an expiration date of 30 days. Store at 4°C.
Freezing medium: DMEM/DMSO	
Preparation	8.87mL of DMEM (Dulbecco's Modified Eagle Medium + 1.0 mL of DMSO + 134 µL of BSA (Bovine Serum Albumin)
Storage	Label as (s-DMEM/DMSO) with an expiration date of 30 days.

	Store at 4°C.
Freezing medium: RLT-BME	
Preparation	1ml RLT + 10µl BME
Storage	Label as RLT-BME with an expiration date of 24 hours. Store at 4°C.
Freezing medium: Modified complete RPMI (mcRPMI)	
Preparation	20 % FBS + 70 % cRPMI + 10 % DMSO
Storage	Label as 2x Freezing Media with an expiration date of 30 days. Store at 4°C.
Freezing medium: 70%IMDM/10%DMSO/20%Human Serum AB	
Preparation	70mL IMDM + 10 mL DMSO + 20mL Human Serum AB
Storage	Label as 70%IMDM/10%DMSO/20%Human Serum AB with an expiration date of 30 days. Store at 4°C.
Freezing medium: CTL cryo-ABC cryopreserve media	
Preparation	– Mix CTL cryo-A with CTL cryo-B in a 4:1 ratio by slowly adding CTL cryo-A slowly into CTL cryo-B. Warm the resulting CTL cryo-AB mix and CTL cryo-C in a 37°C CO2 incubator. – Slowly over a period of 2 minutes add an equal volume of CTL cryo-AB mix and CTL cryo-C containing the PBMCs resulting in intended final cell concentration.
Storage	No label necessary as media is prepared with patient samples. Any extra CTL cryo-ABC media is to be discarded at end of workday.
Freezing medium: 85% F/T-20 medium/15%DMSO	
Preparation	85% F/T-20 medium + 15%DMSO
Storage	Label as 85% F/T-20 medium/15%DMSO with an expiration date of 30 days. Store at 4°C.
90%RPMI/10%FBS	
Preparation	90% RPMI + 10% FBS
Storage	Label as RPMI/FBS with an expiration date of 30 days. Store at 4°C.
Complete RPMI (cRPMI)	
Preparation	86% of RPMI 1640 + 1% 100x Penicillin/Streptomycin + 1% 100x L Glutamine + 2% 1M HEPES + 10% FBS
Storage	Label as cRPMI 1640 with an expiration date of 30 days. Store at 4°C.
1%RPMI (+L) glutamine	
Preparation	1 % FBS + 1% Pen/Strep + 98% RPMI (+)L-glutamine
Storage	Label as 1%RPMI with an expiration date of 30 days. Store at 4°C.

F/T-20 medium	
Preparation	Complete RPMI + 20% FBS
Storage	Label as F/T-20 medium with an expiration date of 30 days. Store at 4°C.
Wash Buffer: PBS + 1mL Human Serum Albumin 20%	
Preparation	500mL PBS + 1mL Human Serum Albumin 20%
Storage	Label as Wash Buffer: PBS + 1mL Human Serum Albumin 20% with an expiration date of 30 days. Store at 4°C.
3% Dextran	
Preparation	Dissolve 6 g of Dextran and 1.7 g of Sodium chloride (NaCl) in 200 mL of deionized water
Storage	Label as 3% Dextran with an expiration date of 30 days. Store at 4°C.
2 N Saline	
Preparation	Dissolve 1.7 g of Sodium chloride (NaCl) in 100 mL deionized water
Storage	Label as 2 N Saline with an expiration date of 30 days. Store at 4°C.

9. Calibration

- N/A for procedure
- All Instruments and equipment should have proper maintenance as specified in the SOP for the instrument or equipment.

9.1. Calibration Information

Calibrator	US Catalog Number	EU Catalog Number	SG Catalog Number	SHA Catalog Number	Vendor
Not Applicable	Not Applicable	Not Applicable	Not Applicable	Not Applicable	Not Applicable

9.2. Calibration Preparation

Not Applicable

9.3. Calibration Frequency

Not Applicable

9.4. Calibration Acceptability

Not Applicable

9.5. Analytical Measurement Range

Not Applicable

10. Quality Control and Batch Acceptance Criteria

- All PBMC technologists must perform a competency assessment at a minimum twice a year and after completing training.

- Fresh whole blood is to be collected day of competency. The whole blood is to be transferred over to a 15ml conical tube so an accurate volume can be recorded.
  - Blood donors are to follow PPD IRB protocol.
  - Alternatively, residual samples can be used. The criteria when using residual samples will need to be adjusted to match the processing of this aged samples. Recovery after thawing should ideally be more than 70%.
- All technologists are to meet the technologist qualifications listed below. "PBMC competency assessment form A" **or** "PBMC competency assessment form B "and "PBMC competency assessment calculation sheet by hematology analyzer" are to be completed at time of competency and handed to the supervisor of the laboratory. For technologists who are able to perform manual cell counts, they will have to fill PBMC competency assessment calculation sheet by hemocytometer counting.
- All employees once completed the competency or training must complete the "PBMC Training Checklist".

- Technologist qualification
  - Technologists must meet the below specified quality parameters.
  - A cell count and a differential are to be taken pre and post isolation and recovery should be calculated.
    - Recovery should be based on:
      - The total percentage of lymphocytes and monocytes from whole blood used. This percentage is to be used to determine the expected yield of PBMCs.
      - Actual recovery should be no less than 25% of the expected recovery for PBMCs due to manual nature of PBMC isolation.
      - Pre isolation cell counts are to be taken from the hematology analyzer. Post isolation cell counts are to be taken by the hematology analyzer and manually counted with a hemocytometer. The results are to be within 20% of one another.
  - Mononuclear cells should not be less than 85% of total automated or manual hematology differential on the PBMC (Neutrophils, Eosinophils, and Basophiles should not exceed 15% of total differential).
  - Viability should be no less than 90% if competency is completed on the whole blood of a healthy donor that was drawn within 2 hours of the start of the competency assessment.
  - Only 1 type of worksheet is needed to be done to demonstrate the competency.
  - Other aspects of competency will be evaluated as well. **This is thawing after storage.** The acceptable criteria for being qualified and pass competency if viability after thawing >90% and recovery after thawing is >70%. When residual samples were being used the viability after thawing >70% and recovery after thawing >70%. Use Table A for the thawing procedure worksheet.
- A QC sterility check for reagents that are open and not normally depleted within one month will be applied to two Thioglycollate Broth tubes:

- 1mL of reagent will be transferred to each tube with a sterile pipette and one of the tube will be placed in an anaerobic micro-incubator and the other one will be placed in an aerobic micro-incubator to determine sterility of the open reagent.
- Gently agitate the reagent to assure proper mixing and assurance microorganisms collected are present.
- Do not tighten the screw cap lids on the Thioglycollate Broth after the placement of the reagent for incubation at 37°C for 48hrs. Leave them slightly loose.
- Thioglycollate Broths are to be observed at 48 hours: any cloudiness is sign of bacterial growth. **If the Thioglycollate Broth shows signs of bacterial growth (cloudiness) the media tested is to be disposed of immediately.**
- **During QC sterility check, reagents are kept aside. Not to be used until testing is finished.**
- "Reagent QC log" attached below is to be completed. The QC sterility check is to be performed 1x per month until expiration date and/or depletion of the reagent. For reagents without expiration date from manufacturer please refer to SOP GCL-LAB-0090

- **Monthly QC checks**
  - Spot check of the worksheets will be done at random by PBMC leads or supervisors.
  - This will be done on 3 to 5 accessions or lesser or more depending on the volume of samples per month.
  - Selected worksheets are checked against the master copy in the PBMC content server where all the forms are located to ensure the correct versions are used.
  - Any discrepancies, alert Laboratory management and respective PM.
  - An impact assessment will need to be done to determine the severity of the non-compliance.

- **Aliquot Review**
  - Aliquot review needs to be performed before handing over the PBMC aliquots to Sample Management team for storage in Liquid Nitrogen. The Aliquot review report provide the list of all aliquots created by GCL Lab the previous day. The Technologist routing the PBMC aliquots will use this file to cross check the aliquots he/she is routing and a second Technologist signs the document to ensure all PBMC aliquots go to the correct storage location.
  - Alternatively, aliquots can be routed in ConneXion to Sample Management for audit trail. Once routed, samples are kept in frozen in a designated freezer for Sample management team to store. Aliquot review report is checked the following day to ensure all aliquots are in storage or routed by a second technologist and document accordingly.

10.1. Quality Control Information

Quality Control	US Catalog Number	EU Catalog Number	SG Catalog Number	SHA Catalog Number	Vendor
Not Applicable	Not Applicable	Not Applicable	Not Applicable	Not Applicable	Not Applicable

10.2. Quality Control Preparation

Not Applicable

10.3. Quality Control Frequency

Not Applicable

10.4. Quality Control Acceptability

Not Applicable

11. Equipment and Materials

11.1. Equipment

- Beckman-Coulter LH-750 or equivalent, validated hematology analyzer
  - Cell counts in manual/single mode
- 37°C 5% CO2 incubator
- 37°C Water Bath
- Biologic laminar flow cabinet
  - Class IIA
- Centrifuge – room temperature
  - Horizontal buckets capable of 50 mL tubes
  - Capable of 650 x g; 318 x g; 234 x g
  - Capable of setting “no brake”
- ThermalTray HP
  - Biocision BCS-104

11.2. Materials

- Hemacytometer, Neubauer
  - VWR 15170-172 or equivalent (Disposable 82030-468)
- Cryovials 1.2 mL - Sterile
  - Corning 430658 or equivalent
  - Externally threaded, polypropylene, silicone washer
- Cryovials 3.6 mL - Sterile
  - Corning 366524 or equivalent
- Cryovials 2 mL - Sterile
  - Corning 430659 or equivalent
- Nunc 1.8mL Externally-threated universal tubes
  - Thermofisher scientific #374502
- Centrifuge tube 1.5mL W/ orange cap
  - Corning 430909 or equivalent
- System 100™ tubes 1.5mL
  - VWR 479-3225
- 0.22µm filter system
  - Nalgene, Thermo Scientific, catalog #569-0020 or equivalent
- Cell strainer
  - BD 70 µm sterile
- Mr. Frosty Freezing Containers or equivalent

- Certified freeing rate approximately 1°C per minute
- 30mL Syringe with Luer-Lok tip
  - BD #302832 or equivalent
- Gauge needle: Hamilton Metal Hub Blunt Point needles
  - Hamilton #91018
  - Fisher #14-815-606 or equivalent
- Plastic transfer pipettes Phoenix: PP-137-030
  - Fisher # NC0221960 or equivalent
- 15 mL conical tubes
  - VWR #5250153 or equivalent
- Polystyrene storage tubes (12x75mm)
  - Fisher #14-961-10 or equivalent
- Caps for 12x75mm polystyrene storage tubes
  - VWR #60819-003 or equivalent
- Glass Pasteur pipettes
  - Fisher # 13-678-20A or equivalent
- Standard Laboratory Supplies:
  - Sterile 15 mL, 50 mL polypropylene tissue culture grade tubes
  - Sterile serologic plastic pipette 1 mL, 5 mL, 10 mL 25 mL
  - Adjustable speed pipet aide (Drummond)
  - Pipette with sterile tips
  - Microfuge tubes for hematology analyzer
  - Parafilm
  - Liquid Nitrogen Dewar's for specimen storage with alarm. Liquid nitrogen tank for replenishment of Dewar's
    - (Note in Belgium it is not allowed to have LN storage tank on site. We have a vendor that refills the Dewar's on regular basis. We will have a second Dewar's container for LN that will be used to fill aluminum tray during scanning samples for shipping)

12. Assay Procedures

NOTE:

- The procedure below is the standard PBMC processing. Please check the PBMC content server for any study specific worksheets.
- However, troubleshooting steps are generic and can be applied across all worksheets.
- For all procedures, use good tissue culture technique keeping all supplies and reagents sterile. Assure that all reagents are sterile (no visible evidence of contamination) and that all plastic ware is sterile.

- **PBMC/BMMC Isolations**
  - ***PBMC Isolation of CPT – Pre- washing steps ("WPD_STANDARD_CPT_PBMC")***
    - Estimate the total volume of whole blood received.

- Record approximate volume
- Gently invert tubes 8 to 10 times
- Centrifuge tube/blood sample at room temperature (18 – 25 °C) in a horizontal rotor (swing-out head) for a minimum of 30 minutes at 1800 RCF (2800 rpm). Please refer to attachment "Centrifuge Nomogram". WARNING: Excessive centrifuge speed (over 2000 RCF) may cause tube breakage. To calculate the correct centrifuge speed for a given RCF, use the following formula:

$$\mathrm{RPM} = \sqrt{\frac{\mathrm{RCF} \times 100000}{1.12 \times \mathrm{r}}}$$

- If collecting plasma gently remove the plasma without disrupting the cell layer.
- With a gently swirling motion collect the buffy coat.
- Collect cell layer with a Pasteur pipette and transfer the collected cells to a 15 mL (for up to two parents tubes) or 50 ml (for 3+ parent tubes) size conical centrifuge tube with a cap. Do not combine different tube types. Collection of cells immediately following centrifugation will yield best results.
- It is recommended by the manufacturer that the CPT tube be spun after 15 minutes of collection.

- ***PBMC Isolation of non-CPT whole blood– Pre- washing steps ("WPD_STANDARD_PBMC")***

- Estimate the total volume of whole blood received.
- Match collection tubes from same patient, verify patient identifiers
- Gently invert tubes 8 to 10 times. Let parent tube sit for two minutes to allow blood to remove from the siding of the parent tube.
- **If collecting plasma**, spin whole blood tube for 10 minutes at 400 RCF at room temperature with a break level at 4. Remove plasma and replace with PBS.
- **If not collecting plasma**, remove whole blood with a sterile plastic pipette and transfer to a 50 ml size conical centrifuge tube (1 parent tube per 50 ml conical centrifuge tube).
- Dilute the blood with equal volume of PBS. Carefully mix contents by pipetting up and down 5 times.
- Add Ficoll-Paque to a separate 50 ml conical tube. Set up 1 conical tube per one blood/PBS mixture. The ratio of blood to Ficoll-Paque Plus should be 2:1 – 2 mL of diluted blood for every 1 mL of Ficoll-Paque Plus (example 30 mL of diluted blood + 15 mL Ficoll-Paque).
- Carefully overlay the Ficoll-Paque Plus with the diluted blood
- As you overlay the additional tubes, some separation by gravity may begin to occur this is acceptable but keep to a minimum. If separation starts to occur begin centrifugation step.
- Overlay only 1 subject at a time to reduce the risk of cross-contamination

- Centrifuge at 900 RCF for 30 minutes at room temperature. The brake should be off.
- Carefully remove the cells at the interface; Recover the PBMC interface using circular motions as it is withdrawn using a 5 mL sterile pipette and transfer the collected cells to another 50 mL conical tube. If the starting tube types are the same, they can be combined into one 50 ml conical tube.
- If collecting PMNC (granulocytes), transfer packed-cell layer or PBMC depleted buffy coat to a 50ml conical tube.
- Continue with red blood cell bulk lysis adding 5 volumes of 1X RBC lysis buffer, incubate 5 mins at room temperature with gentle mixing
- Wash with 1X PBS and repeat if PMNC pellet is contaminated with RBCs.

- ***BMMC Isolation – Pre- washing steps ("WPD_STANDARD_BMMC")***
  - Estimate the total volume of whole blood received.
  - Gently invert tubes 8 to 10 times. Let parent tube sit for two minutes to allow bone marrow to remove from the siding of the parent tube.
  - If collecting plasma, spin whole blood tube for 10 minutes at 400 RCF at room temperature with a break level at 4. Remove plasma and replace with PBS.
  - If not collecting plasma, dilute the bone marrow specimen with PBS with a 1:3 ratio. This is to be done in a 15 ml or 50 ml conical tube depending of volume of bone marrow aspirate received. Up to 4 ml of BM specimen is to be completed in a 15 ml conical tube. 5ml or more is to be completed in a 50 ml conical tube.
  - Pass the diluted BM through a 70 µm mesh filter to remove bone fragments and rinse with 4 mL of PBS to a total volume of 20 mL. Carefully mix contents by pipetting up and down 5 times.
  - Add 15 mL of Ficoll Paque Plus to 50 mL conical tubes (one 50 ml conical tube per every 20 ml of BM+PBS mixture).
  - Carefully layer the 20mL of the diluted BM onto the Ficoll Paque Plus, careful not to disturb the interface.
  - Spin tube for 30 minutes at 900 RCF at room temperature. No Brake.
  - Carefully remove the cells at the interface; Recover the BMMC interface using circular motions as it is withdrawn using a 5 mL sterile pipette and transfer the collected cells to another 50 mL conical tube.

- ***PBMC and BMMC Isolation (Both for CPT and non-CPT): Washing, lysing, cell and viability counting.***
  - Each conical tube with the collected cells is to be filled up to the 15 ml or 50 ml mark (depending on what conical tube the cells are collected in) with PBS.
  - **Washing step 1**- Spin at 400 x g for 5 minutes; Brake at level 4.
  - **Washing step 2** - Carefully and slowly dump the supernatant off. DO NOT DISRUPT THE CELL PELLET. Do not invert the tube. Keep the tube face down.

- **Washing step 3** - Blot the face of the tube with a gauze sponge that is kept in sterile conditions in the biological safety cabinet.
- **Washing step 4** - Slowly tilt the conical tube facing up and place in a rack inside the biological safety cabinet.
- **Washing step 5 –** Re-suspend the pellet by moving the conical tubes across the hood grates and following up by gently flicking with your finger.
- If the pellet is bloody, add 3-5 ml of RBC lysing solution to conical tube. Let it incubate on ice for 5 – 10 minutes.
- Fill the remaining space of the conical tube with PBS.
- Repeat the steps for washing as stated above one additional time for patients that were RBC lysed.
- After washing is completed, re-suspend the pellets in 6 ml of PBS.
- Remove 300 µl and place in a 12 x 75 mm plastic polystyrene or polypropylene culture tube. Cap with an appropriate plastic closure plug. Cell counts and viability are to be completed from this aliquot. **WARNING: The cell counting and viability is to be completed immediately. Prolonged time may result in a false low cell count and viability.**
    - Refer to SOP GCL-LAB-0628 for viability instructions.

- **Sample Aliquoting**

  Per clients request the options available for freezing media are listed at the start of **8.2 Reagent Preparation.** Isolates may also be dry pelleted.

- **Sample storage conditions**
  - When ready to freeze add the required amount cold Freezing media. Place appropriately labeled cryovials (pre-cooled to -20°C for 30 minutes) in the ice and add 1 mL of suspension to each tube. Re-cap.
  - Place the cryovials into a charged Mr. Frosty (containing 120 mL of cold isopropanol – the isopropanol is good for **5 freeze cycles**)
  - Place into the -70°C freezer
  - Transfer cryovials to sample management to store in liquid nitrogen after 24 hours or on Monday if cells were processed over the weekend.
  - Log the specimens into the LIS Storage module, as they are transferred to liquid nitrogen. Keep on dry ice as processing.
  - Dry cell pellets (cells pelleted in tubes without reagents can also be frozen via snap freezing in LN2 .
  - Cells can also be suspended in nucleic acid preservative such as RNA protect.
- **Leucocyte (WBC) Isolations**
  - Gently invert tubes 8 to 10 times. Let parent tube sit for two minutes to allow blood to remove from the siding of the parent tube.
  - Obtain a 30 mL syringe and remove the plunger. Make sure the plunger will form a tight seal with the syringe before proceeding

- Invert the syringe while placing thumb or finger over the tip.
- While keeping thumb in place, pour up to 10 mL of whole blood into the syringe (do not exceed 10 mL). It is very important to keep thumb in place so no blood leaks or spills out of the syringe.
- Add 3% Dextran to the syringe using a plastic transfer pipette in a ratio of 4:1 Dextran: blood with a maximum total volume in the syringe of 20 mL. For example, if there is 4 mL whole blood (or packed cells) in the syringe, add 16 mL 3% Dextran. But for any volume of whole blood (or packed cells) greater than 4 mL, add 3% Dextran until the total volume (blood +Dextran) in the syringe is 20 mL.
- Carefully replace the plunger in the syringe while keeping thumb in place. The pressure formed in the syringe will make it difficult to replace the plunger. Great care must be taken that the plunger is replaced without allowing any blood to leak or spill.
- Invert the syringe five to six times to mix the 3% Dextran and blood together.
- With the plunger-side down, carefully remove your thumb from the syringe and replace with a Kimwipe to absorb any blood that escapes due to the built-up pressure.
- Slowly press in the plunger, which will allow small spurts of blood and air bubbles to escape. Absorb these with the Kimwipe. Most of the air bubbles should be removed from the syringe in this manner.
- Pull the syringe back so that there is approximately 1 cm of space between the blood mixture and the tip of the syringe.
- Attach a bent #18 gauge blunt tip needle on the end of the syringe. Allow the syringe to sit upright (needle end up) for approximately 30-45 minutes.
- After 30-45 minutes, the contents of the syringe should be visibly separated into two layers. (Some samples (especially those that have undergone hemolysis) may require longer to separate)
- In a clean, 15 mL polystyrene conical tube. Slowly push in the plunger of the syringe, allowing the upper, clear layer to be pushed out of the needle. Collect this layer in the conical tube. It is important to remove as much of the clear liquid as possible without removing any of the red blood cell mixture. If red blood cells are accidentally collected in the centrifuge tube, extra wash steps may be required (steps 16-19) to remove them.
- Cap the tube and spin this solution in a centrifuge for 10 minutes at 1000 rpm.
- Properly dispose of syringe and remaining blood mixture. Immediately rinse out needle with water.
- After centrifugation, carefully remove the supernatant by suction and discard.
- To the centrifuge tube, add 5 mL of deionized water. Using a Pasteur pipette, break up the pellet, reconstituting with the water. Allow to stand 45 seconds.
- Add 5 mL of 2 N saline and mix.
- Parafilm and spin the solution again for 10 minutes at 1000 rpm.
- After centrifugation, carefully remove the supernatant by suction and discard.
- Add 1.5 mL of deionized water and 1.5 mL of 2N saline. Break up pellet using a Pasteur pipette and reconstitute well.

- If the pellet looks red, repeat step 22 one more time.
- Transfer contents into a labeled12x75mm plastic storage tube. Spin for 10 minutes at 1000 rpm break 4.
- After centrifugation, carefully aspirate supernatant and discard. There should be a white pellet remaining in the bottom of the storage tube.
- Store WBC pellet at -20°C.

- **Dried blood spot**

Pipette 75 µl of blood into each spot of the Whatman® protein saver cards and let it dry at room temperature.

13. Calculations and Result Reporting

13.1. Calculations

- Calculate cell counts using the worksheet specific for the protocol
- % Viability = number of living cells/total cells x 100%
- % Recovery = ((volume that the cells are in [10 mL] x cell count in cells/µL x 10^6 µL/mL)/cells frozen [10^7])) x 100%

Reporting Results -	
Reportable Range	NA
Repeat Criteria	NA
Critical Values	NA
Dilution	NA
Decimals	1
Qualitative Cut-off values	NA
Maximum Dilution	NA
Units	Total cells; % viable
Unit Conversions	NA

13.2. Result Reporting

Report the required data for the protocol in the LIS (i.e. cell counts, viability, number of vials frozen etc.)

Method CV from PI	**Achieved CV During Validation**
NA	*NA*
Repeat Criteria	
NA	*NA*

14. Reference Ranges

See 17. Limitation

15. Procedural Notes or Related Procedures

TROUBLESHOOTING GUIDE

- No definite interface after the separation step
  This occurs for two reasons:
  - First, the centrifugation time was not sufficient. Replace the tube in the centrifuge and centrifuge for an additional 30 minutes. Make a deviation note on the PBMC worksheet.
  - Second, a faint interface can be seen, it is often due to cloudy or opaque plasma that interferes with the visualization of the interface. Should this occur, leave two centimeters of plasma on top of where the interface appears to begin, then collect the interface with the excess plasma.
- First wash step yields small (or no) pellet
  It is probable that too much Ficoll-Paque Plus was collected and that the amount of C-PBS added did not sufficiently dilute the separation medium present in the wash. Divide the volume into two 50 ml conical tubes and fill each to the 50mL mark with C-PBS Repeat the centrifugation. The resulting pellets may be recombined into one tube. Make a deviation note on the PBMC worksheet.
- Clotted blood in original blood collection tubes
  Clotted blood results from incomplete mixing of anticoagulant after blood drawing. Clots will result in lower PBMC yields. If clotting is present in more than half the blood volume, the PBMC procedure should not be performed and the blood should be re-drawn. If only a small amount of the blood is clotted, the technician may proceed with the procedure, but should make a deviation note on the PBMC worksheet of the presence of any clots detected.
- Disruption of the interface layer before collection
  A violent disturbance to the interface layer may occasionally occur (dropping the centrifuge tube or giving it a “hard bump”). If the interface disperses, remove the entire amount of liquid above the remaining interface and place into two 50 ml conical tubes. Dilute the liquid with C-PBS to the 50mL mark. Overlay 25 mL of this dilution over 10 mL of Ficoll-Paque Plus (four tubes) and centrifuge. Make a deviation note on the PBMC worksheet.
- Disruption of the interface layer during processing
  A large drop of blood at the beginning of the layering process may cause disturbance to the interface. If the interface disperses, stop the layering process, pour back all the blood from the pipette into the original container, discard the contaminated 50ml tube, create a new 50 ml tube with appropriate amount of Ficoll-Paque Plus and continue process as described in **PBMC Isolation and Freezing** section of this SOP.

16. Test Delay

Refer to SOP GCL-LAB-0199 process for communicating assay issues (amended results, delay in testing, etc.) to clients and investigators.
If patient collection tubes lack sufficient identifiers proceed with processing the sample and withhold releasing results until labeling issues have been resolved.

17. Limitations

A very standard generic assumption is that there are 1 million white blood cells per ml of blood. This also includes a fresh collection, isolation right away, and a healthy donor. With the time it takes to arrive in the laboratory after collection, the movement of the specimen during shipping, and the temperature changes from the transport, white blood cells will likely decrease. Also, the 1 million white blood cells per ml also include neutrophils, which on average is 60% of the total white blood cell count. PBMC processing is designed to exclude these cells. A quick assumption is that PBMCs will retrieve about 350,000 PBMCs per every 1 ml of blood.

18. References

- Processing of Clinical Trial Blood Samples DP-IMG 1001.3 April 25, 2011 – Client specific SOP
- Freezing and Thawing Clinical Trial Samples DP-IMG 1005.2 April 25, 2011 Client specific SOP
- Operator Qualification for Blood Processing DP-IGM 2011.1 March 11, 2011 Client specific SOP
- Defining Blood Processing Parameters for Optimal Detection of Cryopreserved Antigen-Specific Responses for HIV Vaccine Trials, J Immunol Methods. 2007 April 30; 322(1-2): 57–69.
- Creating Evidence-Based Procedures Out of Established Processes: Validation of Ficoll-Paque ™ Centrifugation for Isolation of Peripheral Blood Mononuclear Cells. May 2013 Precision Bioservices.

Document Number	SOP Title
GCL-EHS-0026rxx	Bloodborne Pathogen Exposure Control Plan (US)
GCL-EHS-0027rxx	Bloodborne Pathogen Exposure Control Plan (EU)
GCL-EHS-0018rxx	Bloodborne Pathogen Exposure Control Plan (SG)
GCL-EHS-0016rxx	Bloodborne Pathogen Exposure Control Plan (SHA)
GCL-EHS-0025rxx	Chemical Hygiene Plan
GCL-LAB-0199rxx	Guidelines for the Communication of Assay Issues to Sponsors, Clinical Teams, and Investigators
GCL-LAB-0090rxx	Reagent Labelling

19. Attachments

a) PBMC competency assessment form A
b) PBMC competency assessment calculation sheet by hematology analyzer
c) PBMC competency assessment calculation sheet by hemocytometer counting
d) PBMC Training Checklist
e) Reagent QC log
f) Centrifuge Nomogram
g) WPD_STANDARD_PBMC
h) WPD_STANDARD_CPT_PBMC
i) WPD_STANDARD_BMMC

j) Table A Thawing r storage worksheet
k) PBMC competency assessment form B
l) EU PBMC Daily Checklist

PPD, Inc.

Procedural Document Approval Certificate

This Procedural Document has been reviewed and approved according to PPD processes.

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